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(54) **DIAGNOSTIC MOLECULAIRE DES INFECTIONS VIRALES**
(54) **MOLECULAR DIAGNOSIS OF VIRAL INFECTIONS**

(57) On a recours à la transcription inverse faisant appel, en guise d'amorces, à des oligonucléotides courts regroupés de séquence aléatoire pour obtenir de l'ADNc à partir d'ARN viral dans un extrait de cellules que l'on soupçonne d'être infectées par des pathogènes à ARN. L'ADNc peut être amplifié au moyen d'une seule procédure de PCR utilisant des sondes spécifiques à deux pathogènes ou plus afin de déceler la présence d'une infection par de nombreux pathogènes à ARN dans les cellules. L'ADN ainsi amplifié peut comprendre du bromo-déoxyuridine qui est un moyen commode et non radioactif de déceler le produit amplifié.

(57) Reverse transcription employing pooled short oligonucleotides of random sequence as primers is used to obtain cDNA from viral RNA in a extract of cells suspected of being infected by RNA pathogens. The cDNA may be amplified in a single PCR procedure employing primers specific for two or more pathogens in order to detect the presence of multiple RNA pathogen infection in the cells. DNA so amplified may incorporate bromo-deoxyuridine which facilitates a convenient and non-radioactive means for detecting the amplified product.

MOLECULAR DIAGNOSIS OF VIRAL INFECTIONS

This invention relates to the detection of the genetic material of RNA pathogens and in particular, multiple RNA respiratory pathogens.

Laboratory testing to identify disease causing pathogens in clinical specimens is necessary in a variety of circumstances. Test results may be useful for guiding physicians in terms of appropriate patient management and medication, gathering information necessary for epidemiology and public health purposes, and research into mechanisms of infections caused by microorganisms.

Conventional laboratory methods of viral diagnosis such as culturing the pathogen, electron microscopy, immunodetection of the pathogen and serology are subject to significant limitations. Such limitations may be brought about by factors such as poor replication of pathogens in culture, requirement for specialized and expensive equipment (e.g. electron microscopy) and, existence of numerous serotypes of a particular pathogen.

Amplification of genetic material derived from a respiratory pathogen provides a sensitive and specific means for detecting such pathogens. Reverse transcription (RT) of viral RNA followed by DNA amplification in the

polymerase chain reaction (PCR) has been shown to be a sensitive diagnostic procedure for detection of several RNA virus respiratory pathogens: Gilbert, L.L., *et al.* (1996) Diagnosis of Viral Respiratory Tract Infections in Children by using a Reverse Transcription-PCR Panel; Journal of Clinical Microbiology 34:140-143. However, such procedures are complex and require a significant length of time to be carried out. This is disadvantageous in a commercial clinical laboratory setting, particularly in situations where the diagnostic procedure must detect multiple RNA pathogens. In addition to upper and lower respiratory tract infections (e.g. common cold, pneumonia), multiple RNA respiratory pathogens may infect a single patient showing symptoms of asthma or exercise-induced bronchoconstricton (EIB).

Summary of Invention

This invention provides a method to obtain RNA pathogen cDNA through reverse transcription (RT) wherein an extract of cells suspected of containing a RNA pathogen is used in a RT reaction in which pooled short oligonucleotides of random sequence are used as a source of RT primer. The RNA pathogen may be a RNA respiratory pathogen.

While the method of this invention may be used to obtain CDNA from the RNA of any pathogen, the method is

particularly suited to the diagnosis of multiple RNA pathogen infections since the RT reaction does not depend upon use of specific oligonucleotide primers. Rather, the use of random oligomers as primers permits reverse transcription of RNA from multiple sources present in a single sample.

Use of random oligomers as RT primers is known for the detection of RNA from single RNA pathogen sources; see: M.H. Vodkin *et al.* (1994) "PCR - Based Detection of Arboviral RNA from Mosquitoes Homogenized in Detergent", BioTechniques 17:114-116; R.F. Meyer *et al.* (1991) "Rapid and Sensitive Detection of Foot and Mouth Disease Virus in Tissues by Enzymatic RNA Amplification of the Polymerase Gene", Journal of Virological Methods 34:161-172; and, D.K. Howe *et al.* (1992) "Use of the Polymerase Chain Reaction for the Sensitive Detection of St. Louis Encephalitis Viral RNA", Journal of Virological Methods 36:101-110. Also, use of random oligomers as RT primers has been demonstrated for the detection of multiple RNA pathogens from non-cellular sources; see: Yu-Li Tsai, *et al.* (1994) "Detection of Polio Virus, Hepatitis A Virus, and Rotavirus from Sewage and Ocean Water by Triplex Reverse Transcriptase PCR", Applied Environmental Microbiology 60:2400-2407.

This invention provides a method to obtain RNA respiratory pathogen cDNA through reverse transcription

(RT) of genetic material obtained from a respiratory source comprising the steps of:

(a) extracting RNA from cells from a respiratory source; and

(b) using RNA from step (a) as a template in a RT reaction in which pooled short oligonucleotides of random sequence are used as a source of RT primer.

RNA respiratory pathogens detectable by the method of this invention include single stranded RNA viruses of which the following are examples: picoronavirus (rhinoviruses and/or enteroviruses); parainfluenza viruses (PIV); respiratory syncytial virus (RSV); influenza viruses A, B and C; and, coronaviruses OC43 and 229E.

Cells from a respiratory source may be cells of any respiratory specimen including nasopharyngeal or tracheal washes and aspirates, bronchoalveolar levage fluid, and lung tissue specimens.

Methods for extracting RNA from cells and cell suspensions are well known in the art, such as the procedures described in: Chomczynski, P. and N. Sacchi (1987) Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction; Anal. Biochem. 162:156-159. A preferred method of RNA extraction by cell lysis is described below.

Methods for performing RT are well known in the art and commercial RT kits are readily available. However, rather than using viral specific oligonucleotide primers, this method employs a pool of random oligomers as a source of the RT primers. The pool of random oligomers should be assembled such that as many of the possible permutations of random sequences are provided in the pool. This is most conveniently done by mixing different oligomers having the shortest possible sequence that will function efficiently as a RT primer. Hexamers will efficiently act as RT primers at normal temperatures (e.g. 37°C) and there are only 4,096 possible permutations. Pools of random hexamers for use as primers are commercially available (e.g. Pharmacia, Montreal, Canada).

This invention also provides a method of amplifying DNA derived from two or more sources by PCR in a single sample. For example, the DNA may be cDNA derived from two or more RNA respiratory pathogens. According to this aspect of the invention, DNA derived from different sources is amplified in a single sample using primers specific for two or more pathogens. Preferably, the primers are selected such that the amplified DNA associated with different pathogens will be of such differing lengths as to be discernible following usual separation procedures such as electrophoresis. PCR amplification procedures and reagents are well known in the art. Combination of the above described method of RT using random oligomers with

multiple PCR amplification in a single sample provides a greatly streamlined yet efficient process for obtaining detectable levels of genetic material derived from multiple RNA pathogens.

This invention also provides an efficient method for amplification of DNA, such as cDNA derived from RNA respiratory pathogens, whereby the resulting amplified DNA may be detected by an immunostaining procedure which does not require the use of radio-labelled probes yet is comparable in sensitivity to the latter procedure. This method facilitates the above described method of multiple PCR amplification in a single sample since its sensitivity permits the detection of product which is present in a minute amount as compared to other amplified product in the sample. The method was described by the inventors in: A Nonradioactive Method for Rapid and Sensitive Detection of Polymerase Chain Reaction Products by use of Bromo-Deoxyuridine (1996) Modern Pathology 9:849-853.

This invention also provides kits comprising reagents for carrying out the aforementioned methods. A kit for performing the above described RT method will comprise a pool of random oligomers for use as RT primers, preferably a pool of random hexamers. When the kit is to be used for carrying out the above described method of multiple PCR amplification, it will comprise RNA respiratory pathogen specific primers. Such a kit will preferably also comprise

a dNTP mixture in which bromo-deoxyuridine (Br-dUTP) is substituted for dTTP. Such a kit would also preferably include an anti-Br-dU antibody and the remainder of a ELISA system whereby amplified DNA containing Br-dU may be readily detected (e.g. by enzyme-chemiluminescence).

Detailed Description of the Invention

Detailed descriptions of embodiments of this invention are set out in the accompanying examples. Unless otherwise stated, autoclaved plasticware (at least 20 min. on dry heat, 30 psi, 132°C) is used, and aqueous solutions contain 0.1% diethylpyrocarbonate (DEPC) as an RNAse inhibitor. To prevent inadvertent exogenous RNAse activity from handling of specimens, investigators should wear latex gloves that are changed several times during the course of the procedure.

RNA extraction. Total RNA is extracted from a thawed cell suspension (e.g., nasopharyngeal swabs, bronchoalveolar lavage fluid) by use of the RNeasy™ spin column, lysis and wash buffers (Qiagen Corporation) as follows: a 300 μL aliquot of the specimen is added to an autoclaved 1.5 mL Eppendorf tube, followed by addition of 300 μL of lysis buffer RLT, 600 uL of 70% ethanol and mixing by agitation on a vortex. This 1.2 mL sample is applied onto the RNeasy™ spin column and centrifuged for 15 s at 10,000 rpm. The flowthrough is discarded. Seven hundred μL of wash

buffer RW1 is added to the spin column, the sample is centrifuged as above and the flowthrough is discarded. The spin column is placed in a new 2 mL collection tube. Five hundred μL of wash buffer RPE is pipeted onto the spin column, and the sample is centrifuged as above, with the flowthrough being discarded. Wash Buffer RPE (500 μL) is pipeted onto the spin column and the sample is centrifuged at full speed for 2 min. The spin column is transferred to a new collection tube and the RNA is eluted by addition of 30 μl of DEPC-distilled, deionized water and centrifugation at 10,000 rpm for 1 min.

In cases where RNA is extracted from tissue specimens (such as frozen lung tissue) the commercially available TRIZOL™ reagent may be used (see United States patent No. 5,346,994.

Reverse transcription (RT). To remove secondary structure from RNA, a 10 μL aliquot from the RNA sample as prepared above is heated to 68°C for 10 min., then quick-chilled on ice. This linearized RNA is added to an Eppendorf tube that contains 10 μL of the following aqueous RT reaction solution at room temperature: 10 mM Tris-HCl (pH 8.3); 50 mM KCl, 5 mM $MgCl_2$, 10 mM pooled deoxynucleotide triphosphates (dNTPs),(Pharmacia) 1 μL of 50 μM random hexamers to act as "universal" primers for RT, 200 units Moloney murine leukemia virus reverse transcriptase, (Pharmacia) and 20 units of RNAse inhibitor (Pharmacia).

RT occurs in the closed Eppendorf tube at 37°C for 60 min., after which time the tube is heated at 95°C for 5 min. to inactivate the reverse transcriptase. The specimen is then placed in ice until used for PCR.

PCR amplification. PCR amplification is carried out in a 50 μL reaction volume containing: all or part of the 20 μL aliquot from the RT procedure; 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 5 mM $MgCl_2$; 0.2 mM dNTPs, in which an equimolar concentration of 5-bromo-2-deoxyuridine triphosphate (Br-dUTP; Sigma) is substituted for deoxythymidine triphosphate; 2 μM of oligonucleotide primers specific to two pathogens and, 2 units of Taq DNA polymerase (Gibco-BRL). Specimens undergo brief centrifugation and are overlayed with mineral oil. PCR cycling conditions on a ROBOCYCLER 40™ (Stratagene) involve 35 cycles, each one consisting of denaturation at 94°C for 1 min., annealing at 55°C for 1 min., and extension at 72°C for 1 min. (10 min. during the last cycle). Samples undergo electrophoresis on 1-1.5% agarose gels that contain 0.5 μg/mL ethidium bromide. This is followed by conventional Southern blotting on nylon membranes (e.g. Hybond-N™ membranes, Amersham) and fixation of any PCR products (DNA) by cross-linking under ultraviolet light for 2-5 min.

Immunodetection of PCR products. Nylon membranes are pre-equilibrated in 0.1 M Tris buffered saline (TBS), pH 7.5 for 5 minutes at room temperature and then incubated

with blocking buffer (0.1 M TBS, pH 7.5 with 5% non-fat dry milk powder (Carnation)) at 37°C for 15 min. Mouse anti-bromodeoxyuridine antibody (Boehringer Mannheim) is added at a final dilution of 1:1,000 in blocking buffer for a 45 min. incubation at 37°C. After three washes of 5 min. each in TBS, the nylon membranes are incubated at 37°C for 30 min. with peroxidase-conjugated goat anti-mouse immunoglobulin diluted 1:2,000 in blocking buffer. After three final washes in TBS, enzyme-chemiluminescent (ECL) substrate (Amersham) is applied and the membranes are exposed to x-ray film with regular intensifying screens for a period varying from 15 sec. to 5 min.

Example 1

Children selected from a survey of primary school children in Port Alberni, British Columbia, Canada, were identified as having asthma, EIB or as controls. Age and sex distribution of the study participants is shown in Table 1. A nasal swab specimen was taken from each child from the left inferior nasal turbinate with the swab being rotated once. Each specimen was stored in a cryovial on ice and was subsequently refrigerated at -70°C pending further processing. Positive controls for RT-PCR were prepared from total cellular RNA extracted from virus infected cell cultures grown in M199 minimum essential medium (Gibco BRL) supplemented with 5% fetal calf serum, L-glutamine and vitamins. All viruses and cell lines were

obtained from American Type Culture Collection. HEp-2 cells were infected with Long strain type A RSV; MRC-5 cells were infected with human rhinovirus, type 1B; Rhesus monkey kidney (MK2) cells were infected with one of: picornavirus, PIV type 3, Influenza A Weiss-43, human coronavirus OC43, and human coronavirus 229E. Negative controls included blank reagent mixtures which contained all constituents except a nucleic acid template, or with a nucleic acid template prepared from total cellular RNA extracted from uninfected cell cultures of the appropriate type.

The above described procedures using a RNeasy™ spin column were carried out for RNA extraction. Total RNA was eluted into 30 μL of 0.1% DEPC containing distilled water. Two 10 μL aliquots of total RNA were incubated for ten minutes at 70°C to remove secondary structure and were quickly chilled on ice. Reverse transcription according to the above described procedures using random hexamers as primers were carried out for each aliquot of linearized RNA.

Five μL aliquots of cDNA from the RT step were added to separate 45 μL aliquots of PCR reaction buffer containing 10 mM Tris-HCl (pH 8.3), 5.0 mM $MgCl_2$, 10 mM pooled dNTPs, 20 U Taq polymerase (GIBCO BRL), and 2 μM of virus-specific oligonucleotide primers as shown in Table 2. PCR was performed on a Robocycler 40™ (Stratagene, La

TABLE 1. Age and sex distribution of study participants

	ASTHMA (n = 21)	EIB (n = 16)	CONTROL (n = 33)
Male/Female	12/9	6/10	14/19
Median age ± SD (years)	11.8 ± 2.0	11.4 ± 2.0	11.4 ± 1.8

TABLE 2. Oligonucleotides used for viral RT-PCR

Virus	Region	Oligonucleotide Sequence (5' - 3')	PCR Product Size (bp)
RSV	nucleocapsid	primer 1 GCGATGTCTAGGTTAGGAAGAA primer 2 GCTATGTCCTTGGGTAGTAAGCCT probe TAGCTCCAGAATACAGGCATGACTC	410
PIV	F gene 5' nontranslated region	primer 1 AGAGGTCAATACCAACAACTA primer 2 TAGCAGTATTGAAGTTGGCA probe AAAATTCCAAAACAGACCGGC	205
Picornavirus	5' nontranslated region of rhinovirus 1B	primer 1 GCACTTCTGTTTCCCC primer 2 CGGACACCCAAAGTAG probe GCATTCAGGGGCCGGAG	380
Influenza A	matrix protein	primer 1 CAGAGACTTGAAGATGTCTTTGC primer 2 GCTCTGTCCATGTTATTTGGATC probe TCCTGTCACCTCTGACTAAGGGGATTTTG	212
Coronavirus 229E	nucleocapsid	primer 1 GGTACTCCTAAGCCTTCTCG primer 2 TGCACTAGGGTTAATGAAGAGG probe GACTATCAAACAGCATAGCAGC	370
Coronavirus OC43	nucleocapsid	primer 1 AGGAAGGTCTGCTCCTAATTCC primer 2 TGCAAAGATGGGGAACTGTGGG probe GGTCTGGCAAAACTTGGCAAGG	450

Jolla, CA). For each virus tested by the panel, 35 PCT cycles were performed, each cycle consisting of 1 min. denaturation of 94°C, 1 min. annealing at 55°C and 2 min. extension at 72°C (10 min. during cycle 35). A PCR experiment consisted of testing 10 patient specimens for one of the six asthma-associated viruses.

After completion of PCR, a 45 μL aliquot of each PCR sample underwent electrophoresis on ethidium-bromide-stained, 1-2% agarose gels (GIBCO BRL), in TBE buffer that contained 44.5 mM Tris-HCl, 44.5 mM boric acid and 1 mM EDTA, Gels were photographed under ultraviolet light using Polaroid 667 ISO 3000 professional print film, after which RT-PCR products underwent Southern transfer onto nylon membranes, hybridization with the virus-specific, ^{32}P-labelled oligonucleotide probes as shown in Table 2, and autoradiography.

Viral RNA was detected in 43/70 (61.4%) of specimens. Influenza A viral RNA was detected in higher proportion of specimens from clinically stable asthmatic children and children with EIB, in comparison to asymptomatic controls. Table 3 summarizes the proportion of subject showing evidence of RNA from each of the viruses examined. Table 4 shows that there were similar proportions of subjects in the three groups who had none, one or multiple viruses detected by the RT-PCR panel. The results reveal a high prevalence picoranviral RNA in the three groups of children. The RT protocol may have yielded more virus related cDNA for PCR amplification since DNA could be synthesized from both the genomic RNA of the virus and from viral mRNA transcripts. The random hexamers acting as primers would permit reverse transcription of both forms of virus specific RNA despite differences in polarity.

Example 2

cDNA samples resulting from the above described RT procedures are subjected to duplex PCR amplification using the primers shown in Table 5. In each duplex reaction, a cDNA sample is exposed to primers for two pathogens whereby amplification of cDNA associated with each of the pathogens occurs simultaneously in a single sample. The following scheme is employed in order that the amplification products will differ significantly in size to facilitate electrophoretic separation:

(a) picornavirus and parainfluenza virus (PIV);

(b) RSV and influenza A virus;

(c) influenza B and C viruses; and

(d) Coronaviruses OC43 and 229E.

Detection of amplification products may be carried out according to the radio labelling procedure of Example 1 using labelled probes as shown in Table 2, or by detection of the incorporation of Br-U in amplified DNA as described above and in Example 3.

Simultaneous detection of DNA respiratory pathogens such as adenovirus and Chlamydia may be carried out. Suitable PCR primers for the latter organisms are shown in Table 5. The procedure employed may involve either RT of total RNA in a sample followed by amplification of cDNA derived from both RNA and DNA pathogens. Alternatively, a DNA preparation obtained from the respiratory source may be mixed with a cDNA preparation obtained from the above described RT step and the single sample is then subjected to multiple PCR amplification of RNA pathogen cDNA and DNA pathogen DNA according to the methods described above.

TABLE 3. Number (percentage) of subjects showing positive RT-PCR results for each virus in the panel.

	ASTHMA (n = 21)		EIB (n = 16)		CONTROL (n = 33)	
RSV	3	(14)	0	(0)	8	(24)
PIV	4	(19)	1	(6)	5	(15)
Picornavirus	8	(38)	4	(25)	8	(24)
Influenza A	7	(33)*	8	(50)*	5	(15)
Coronavirus 229E	2	(10)	0	(0)	2	(6)
Coronavirus OC43	3	(14)	4	(25)	5	(15)

* p = 0.034 in comparison to asymptomatic controls

TABLE 4. Number (percentage) of subjects showing viral RNA detected by the RT-PCR panel

Number of viruses detected by RT-PCR panel	ASTHMA (n = 21)		EIB (n = 16)		CONTROL (n = 33)	
0 viruses detected	8	(38)	6	(38)	13	(39)
1 virus detected	4	(19)	5	(31)	10	(30)
≥ 2 viruses detected	9	(43)	5	(31)	10	(30)

TABLE 5. PCR Primers

Picornavirus (380 bp product)	5' CGGACACCCAAAGTAG 3' 5' GCACTTCTGTTTCCCC 3'
Parainfluenza (205 bp)	5' AGAGGTCAATACCAACAACTA 3' 5' TAGCAGTATTGAAGTTGGCA 3'
RSV (410 bp)	5' GCGATGTCTAGGTTAGGAAGAA 3' 5' GCTATGTCCTTGGGTAGTAAGCCT 3'
Influenza A (212 bp)	5' GCTCTGTCCATGTTATTTGGATC 3' 5' CAGAGACTTGAAGATGTCTTTGC 3'
Influenza B (365 bp)	5' AGCGTTCCTAGTTTTACTTGCAT 3' 5' GAAAAATTACACTGTTGGTTCGG 3'
Influenza C (425 bp)	5' GCCAGTAATACCAGCAATCTCG 3' 5' CCCTAATGTCTTGGAGAAGCCAC 3'
Coronavirus OC43 (450 bp)	5' TGCAAAGATGGGGAACTGTGGG 3' 5' AGGAAGGTCTGCTCCTAATTCC 3'
Coronavirus 229E (370 bp)	5' TGCACTAGGGTTAATGAAGAGG 3' 5' GGTACTCCTAAGCCTTCTCG 3'
Adenovirus (300 bp)	5' CAGCACGCCGCGGATGTCAAAGT 3' 5' GCCGCAGTGGTCTTACATGCACATC 3'
Chlamydia (145 bp)	5' GCCTGTAGGGAAT(CG)CAGCT(CG)AA(CT)CA 3' 5' GTCGAAAACAAAGTC(AT)CC(AG)TAGTA 3'

Example 3

The above described PCR procedures are carried out using the method described by the inventors at: (1996) Modern Pathology 9:849-853 in which dTTP is substituted by Br-dUTP in the PCR reaction followed by electrophoresis, Southern Blotting and immunostaining as described above. Duplex RT-PCR for RSV and influenza A virus in the presence of Br-dUTP was carried out with a constant amount of RSV and serial 10-fold dilutions of influenza A virus. The results yield a distinct influenza A band (212 bp product) that may be detected in specimens in which 1:1000 of the starting amount of viral RNA was mixed with a large excess of RSV RNA (410 bp product).

As will be apparent to those skilled in the art in the light of the foregoing disclosure, many alterations and modifications are possible in the practice of this invention without departing from the spirit or scope thereof. Accordingly, the scope of the invention is to be construed in accordance with the substance defined by the attached claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The University of British Columbia

(ii) TITLE OF INVENTION: MOLECULAR DIAGNOSIS OF VIRAL INFECTIONS

(iii) NUMBER OF SEQUENCES: 28

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(F) POSTAL CODE: V6B 4N8

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGATGTCTA GGTTAGGAAG AA 22

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTATGTCCT TGGGTAGTAA GCCT 24

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TAGCTCCAGA ATACAGGCAT GACTC 25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGAGGTCAAT ACCAACAACT A 21

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TAGCAGTATT GAAGTTGGCA 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAAATTCCAA AACAGACCGG C 21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCACTTCTGT TTCCCC 16

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGGACACCCA AAGTAG 16

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCATTCAGGG GCCGGAG 17

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAGAGACTTG AAGATGTCTT TGC 23

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCTCTGTCCA TGTTATTTGG ATC 23

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TCCTGTCACC TCTGACTAAG GGGATTTTG 29

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGTACTCCTA AGCCTTCTCG 20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGCACTAGGG TTAATGAAGA GG 22

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GACTATCAAA CAGCATAGCA GC 22

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGGAAGGTCT GCTCCTAATT CC 22

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGCAAAGATG GGGAACTGTG GG 22

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGTCTGGCAA AACTTGGCAA GG 22

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AGAGGTCAAT ACCAACAACT A 21

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TAGCAGTATT GAAGTTGGCA 20

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGCGTTCCTA GTTTTACTTG CAT 23

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GAAAAATTAC ACTGTTGGTT CGG 23

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GCCAGTAATA CCAGCAATCT CG 22

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCCTAATGTC TTGGAGAAGC CAC 23

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CAGCACGCCG CGGATGTCAA AGT 23

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GCCGCAGTGG TCTTACATGC ACATC 25

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GCCTGTAGGG AATSCAGCTS AAYCA 25

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GTCGAAAACA AAGTCWCCRT AGTA 24

WE CLAIM:

1. A method to obtain cDNA derived from multiple RNA respiratory pathogens present in cells from a respiratory source, comprising the steps of:

(a) extracting RNA from the cells;

(b) mixing RNA from step (a) with pooled short oligonucleotides of random sequence in the presence of reverse transcriptase and deoxynucleotide triphosphates to produce cDNA;

(c) mixing cDNA obtained in step (b) with oligonucleotide primers specific to two or more RNA respiratory pathogens in the presence of deoxynucleotide triphosphates, and DNA polymerase; and

(d) performing a polymerase chain reaction by altering conditions so as to repetitively denature, anneal, and extend DNA thereby producing multiple copies of cDNA obtained in step (b) having sequences hybridizable to said oligonucleotides specific to respiratory pathogens.

2. The method of claim 1 wherein the deoxynucleotide triphosphates in step (c) comprise 5-bromo-2-deoxyuridine triphosphate and the method includes the additional step of

detecting bromodeoxyuridine containing DNA with a anti-bromodeoxyuridine antibody.

3. The method of claim 1 or 2, wherein the pooled short oligonucleotides of random sequence are hexamers.

4. A kit comprising a pool of short oligonucleotides of random sequence for use in reverse transcription; and, oligonucleotides hybridizable to unique cDNA sequences corresponding to RNA of two or more respiratory pathogen.

5. The kit of claim 4 wherein the short oligonucleotides of random sequence are hexamers.

6. The kit of claim 4 or 5 further comprising a reverse transcriptase and a DNA polymerase.

7. The kit of claim 4, 5 or 6, further comprising deoxynucleotide triphosphates for use with the reverse transcriptase, and deoxynucloetide triphosphates which comprise 5-bromo-2-deoxyuridine triphosphate for use with the DNA polymerase.

8. The use of pooled short oligonucleotide of random sequence in a reverse transcription reaction to obtain RNA pathogen cDNA from RNA obtained from an extract of cells suspected of containing RNA from two or more RNA pathogens.

ABSTRACT

Reverse transcription employing pooled short oligonucleotides of random sequence as primers is used to obtain cDNA from viral RNA in a extract of cells suspected of being infected by RNA pathogens. The cDNA may be amplified in a single PCR procedure employing primers specific for two or more pathogens in order to detect the presence of multiple RNA pathogen infection in the cells. DNA so amplified may incorporate bromo-deoxyuridine which facilitates a convenient and non-radioactive means for detecting the amplified product.